ASSOCIATION BETWEEN PHYSICOCHEMICAL PARAMETERS AND PRESENCE OF PATHOGENIC INDICATOR MICROORGANISMS IN MUD CRAB

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Association between Physicochemical Parameters and Presence of Pathogenic Indicator Microorganisms in Mud Crab

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A thesis submitted in partial fulfilment of requirement for degree of Bachelor of Science with Honours (Resource Biotechnology)

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DECLARATION

I hereby declare that this thesis entitled "Association between physicochemical parameters and presence of pathogenic indicator microorganisms in crab" submitted to the faculty of Resource Science and Technology is a presented of my original work except for the citations and references and never been before or concurrently submitted for any other degree qualification or other institutions. This work was submitted to partially fulfil the requirement for the degree of Bachelor of Science with Honours in Resource Biotechnology at Universiti Malaysia Sarawak.

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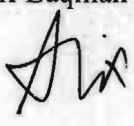
Signature : 

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celcius
µL	Microlitre
APC	Aerobic plate count
APW	Alkaline peptone water
Cfu/mL	Colony forming unit per Mililiter
EMB	Eosine Methylene Blue
LB	Luria-Bertani
$MgCl_2$	Magnesium Chloride
mL	Mililiter
pH	Measure of alkalinity or acidity
Rpm	Revolutions per minute
sdH_2O	Sterile distilled water
TCBS	Thiosulphate-citrate-bile Salts-sucrose
XLD	Xylose Lysine Deoxycolate

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Association between Physicochemical Parameters and Presence of Pathogenic Indicator Microorganisms in Mud Crab

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ABSTRACT

The incidence of foodborne illness have increased globally in recent years, which could be partly attributed to environmental pollution. Changes in environment from their natural state may have an effect on pathogenic microbial content in seafood. This project was done in order to study the relationship between selected physicochemical parameters of local habitats of mud crab and presence of three pathogenic indicator bacteria. Thirteen mud crabs from three different sources were sampled then tested for their Aerobic Plate Count (APC) and specific detection using PCR for the presence of *Salmonella typhimurium*, *Vibrio cholerae*, and *Escherichia coli* O157:H7. Temperature, pH, Dissolved Oxygen (DO) and Biochemical Oxygen Demand (BOD) of their habitats were also tested. Out of three sample's sources, crabs from Pendam River, Asajaya has the least favorable level of DO and BOD according to the national standard and also possess the highest plate count, which were for presumptive *S. typhimurium* was $5.0x\ 10^6$ CFU/mL, *V. cholerae* is 5.2×10^6CFU/mL and *E. coli* is 4.4×10^7 CFU/mL. However, when tested with PCR, there were no presence of these targeted species. In conclusion, there was an association between physicochemical parameters of water and presence of pathogenic indicator microorganisms in crab.

Keywords: Physicochemical parameters, *Salmonella typhimurium, Vibrio cholerae, Escherichia coli*, CFU/mL, mud crab

ABSTRAK

Kejadian penyakit bawaan makanan semakin meningkat di peringkat global dalam beberapa tahun yang lepas, sebahagiannya mungkin berpunca dari pencemaran alam sekitar. Perubahan dari keadaan asal alam semula jadi ini mungkin memberi kesan terhadap kandungan mikrob pembawa penyakit dalam makanan laut. Projek ini telah dijalankan untuk mengkaji hubungan antara parameter fizikokimia habitat ketam nipah tempatan dan kehadiran tiga bakteria penanda berbahaya. Tiga belas ketam nipah dari sumber yang berbeza telah disampel dan kemudian diuji dengan Plat Kiraan Aerobik (APC) dan pengesananan secara spesifik menggunakan kaedah PCR untuk mengesan Salmonella typhimurium, Vibrio cholerae, dan Escherichia coli O157:H7. Suhu, pH, 'Dissolved Oxygen' (DO), dan 'Biochemical Oxygen Demand' (BOD) habitat mereka turut diuji. Daripada tiga sumber sampel, ketam dari Sungai Pendam, Asajaya mempunyai tahap DO dan BOD paling kurang baik mengikut standard nasional, dan kiraan plat tertinggi, iaitu sangkaan S. typhimurium sebanyak $5.0x\ 10^6$ *CFU/mL, V. cholerae* 5.2×10^6 *CFU/mL dan E. coli* 4.4×10^7 *CFU/mL. Walaubagaimanapum, apabila diuji dengan PCR, kehadiran spesis yang disasarkan tidak dikesan. Kesimpulannya, memang terdapat hubungan antara keadaan parameter fizikokimia air dan kehadiran mikroorganisma penanda berbahaya di dalam ketam.*

Kata Kunci: Parameter fizikokimia, Salmonella typhimurium, Vibrio cholerae, Escherichia coli, CFU / mL, ketam nipah

1. INTRODUCTION

Foodborne diseases is a serious public health issue either globally or locally due to their high occurrence and momentous impact on economic and trade in both developing and developed countries including Malaysia. Even in this 21st century, the outbreak of foodborne illness is still very challenging to be reduce. In Malaysia, although the true number of cases for foodborne disease is not fully reported, however the incidence is low ranging from 1.56 to 0.14 cases per 100,000 population (Ezat *et al.*, 2013). Food borne disease can be define as any illness caused by the consumption of foods or refreshments that are contaminated by one or more pathogenic agents. Seafood is responsible for an important proportion of food-borne illness and outbreaks worldwide (Iwamoto *et al.*, 2010). Although they are one of the main sources of protein, high in iodine, vitamin D, selenium and omega-3 oils, there are potential for them to carry pathogenic agent as they can obtain pathogens from various source including from their contaminated marine environment (Ezat *et al.*, 2013). Seafood consist of echinoderms, molluscs and crustaceans. Crabs, just like other seafood, have the possibilities to cause foodborne illness.

Food contamination may take place in any stage from food production process to consumption and in some cases it can also cause by environmental contamination. In seafood case, some seafood possess higher risk of carrying pathogenic agent than others due to many factors such are the environment nature from which they originate, feeding mode, harvesting season and how they are prepared and served (Iwamoto *et al.*, 2010). There were also a research by Popovic *et al* (2010) stating that seafood harvested from polluted waters have an important role in transmitting food borne illness to human. The habitat of seafood or the environment from which they originate can be contaminated with human sewage. Overboard sewage discharge into harvest areas, illegal harvesting from sewage-contaminated waters,

and sewage runoff from points inland after heavy rains or flooding have been identified as a sources of seafood contamination (Iwamoto *et al.*, 2010). It is crucial to study the physicochemical parameter of the seafood's habitat to know whether the habitat is contaminated or not. The physicochemical parameters which can affect the microbial quality of seafood are dissolved oxygen and biochemical oxygen demand

The problem that were emphasised in this project that research on microbial quality of seafood and their habitat condition does not given a special interest as it deserved. They deserved special interest because seafood is one of the important sources of protein and a large number of illnesses due to pathogenic bacteria in seafood consumed will have a significant economic impact on the country. It is also important because in order to provide confident on microbial quality in seafood and to acknowledge the risk of consuming contaminated seafood for the safety of human consumption. This project is also important in order to increase the awareness on the importance of keeping the environment clean in crab growing area. The hypothesis is crabs from more polluted sites will have higher amount of pathogenic indicator microorganism.

The objectives of this project are:

1. To determine the physicochemical parameters of selected water habitat of crab.
2. To detect and enumerate the presence of pathogenic indicator microorganisms in the crab's muscles.
3. To investigate the relationship of physicochemical parameters and the presence of pathogenic indicator microorganisms in crab.

2.0 LITERATURE REVIEW

2.1 Physicochemical Parameters of water

Physicochemical parameters are instrumental methods that involve physical and chemical tests on water sample. It is important know the goal of using the water in order to select suitable parameters for testing of water (Patil *et al.*, 2012). The quality of the water body could influence the quality and variety of the microorganisms exists on the surface of seafood and fishery product (Grisi & Gorlach-Lira, 2010). Some factors that could change the physicochemical parameters of water bodies are from land use change either legal or illegal development, poor solid waste management, uninhibited river water abstraction in upstream, local community that live near to water body have low awareness for importance of clean water, development that are not properly planned, and administration that are not competent (Khalik *et al.*, 2013). Crustaceans, such as crab, are very sensitive to pollutions and the physicochemical parameters influence highly on the dispersion of crabs (Varadharajan *et al.*, 2013). The physicochemical parameters of water also carry an important task in ecosystem health. The most prominent parameters of water in terms of effect on aquatic ecosystems include acidity, salinity, temperature, Total Dissolved Solids (TDS), pH, dissolved oxygen (DO) and biochemical oxygen demand (BOD) (Yazdian *et al.*, 2014). The physicochemical parameters are different between different sites which have different pollution rate such as polluted area have lower salinity and dissolved oxygen (Grisi & Gorlach-Lira, 2010). Either than that, some of the physicochemical parameters are sensitive toward temperature (Yazdian *et al.*, 2014).

2.1.1 pH

A lot of important information about biological and chemical process of natural water can be obtained through studying their pH. In unpolluted water, the pH is influenced with the Carbon Dioxide, CO2, with the atmosphere (Toews, *et al.*, 1995). To determine the corrosive nature of water, pH is the most crucial parameter. The increase of corrosive nature of water is caused by the decrease of pH. Fluctuation of pH can be an indicator for the presence of industrial pollutant, photosynthesis or the respiration of algae that is feeding on contaminant. It is believed that the more polluted the water body, the lower the pH. When rate of photosynthesis are decreased, the pH will increase (Patil *et al.*, 2012). Most industrialized countries has been integrating pH monitoring into the environmental laws. According to the Ministry of Health Malaysia (2004), the acceptable value of pH for raw water is between 5.5 to 9.0.

2.1.2 Temperature

Temperature affects a lot of aquatic organisms and other parameters. It can be fatal if they changed drastically. Water temperature control the rate of all chemical reactions and also marine organism's growth, immunity and reproduction (Patil *et al.*, 2012). Temperature is rather easy to measure. Naturally water bodies will show changes daily or seasonally. However, urbanization and agricultural activities may cause man-made changes toward temperature of the streams (Hancock, 2002). Under certain environmental stress condition, such as changes in temperature may cause seafood to be susceptible to infectious disease cause by pathogens (Snieszko, 2006). The acceptable temperature for raw water is 2°C between the normal temperatures (Ministry of Health, 2004).

2.1.3 Dissolved Oxygen

Among other parameters, dissolved oxygen is one of the most important out of them. Its interrelationship with water body gives direct and indirect information such as photosynthesis, bacterial activity and nutrients availability (Patil *et al.*, 2012). Oxygen have the ability to be dissolved in water and dissolved oxygen will balance with the one in atmosphere (Patil *et al.*, 2012). Dissolved oxygen can be affected by temperature and the biological activity such as photosynthesis and digestion of contamination by microorganisms (Best *et al.*, 2007). Increase in temperature will cause the oxygen to be less soluble. During the day, some aquatic plant will conduct photosynthesis thus raising the dissolved oxygen level. Oxygen is needed by microorganisms in water bodies to digest man-made contamination and natural organic material such as fertilizers, suspended material, or petroleum waste. The oxygen are needed to facilitate their digestion process (Best *et al.*, 2007).

2.1.4 Biochemical Oxygen Demand

Determining how organic matter give impact toward the concentration of dissolved oxygen (DO) in a water body is necessary for water quality management. Usually to measure biochemical oxygen demand, the difference of dissolved oxygen between five days is calculated. In 1908, Biochemical Oxygen Demand was chosen to be an indicator of the organic pollution of rivers by the United Kingdom Royal Commission on river pollution (Jouanneau, 2014). Biochemical oxygen demand is a measure of organic material contamination of water. The unit for biochemical oxygen demand is mg/L. By definition, biochemical oxygen demand is amount of dissolved oxygen needed for the biochemical decomposition of organic compound and the oxidation of certain inorganic materials (Patil *et al.*, 2012).

2.2 Pathogenic Indicator Microorganisms

Indicator microorganism can be any of the microorganisms that can be easily cultured that may be found in the intestine and can be used to detect fecal contamination in water analysis. The presence of these microorganisms in a sample means that pathogen might also be there (Cowan, 2012). They are very useful as they provide proof of the existence or absence of disease causing organism living in similar conditions. By looking first for certain indicator organisms which indicate pathogenic organism might also be present, the tiresome and time consuming task of directly detecting the presence of the intestinal pathogens (Gerba, 2009). A standard indicator microorganisms suggested to have these characteristics (Gerba, 2015):

- useful for water of all type
- present whenever enteric pathogens are present
- have a fairly longer survival time than the hardest enteric pathogen
- should not grow in water
- easy to perform testing method
- their density should have some direct relationship to the degree of fecal pollution
- a member of intestinal microorganisms of warm-blooded animals

However, none of the indicator microorganisms fulfil these criteria. Hence, a number of group of microorganisms have been proposed and used as indicator organisms (Gerba, 2015). Some nations in the world have stated the microbial standard of seafood. The amount of indicator microorganisms should be in crustacean shellfish are less than 100 cells/gram (ICMSF, 1986). Some studies on isolation of microbial communities from different organs of crabs from its habitat showed the presence of variety of microorganisms on different organs (Mahalaxmi *et al.*, 2013).

2.2.1 *Escherichia coli*

Escherichia coli was first identified in 1885 from the excrement of healthy individuals. They are gram negative and prevalently facultative anaerobe of the human intestinal microflora (Liu *et al.*, 2015). They are commonly used as indicator for fecal contamination in seafood. Although most of them are non-pathogenic, there are still some strain that acquired different form of genes through numerous ways in the environment that caused to the increase of their pathogenicity such as *E. coli* O157:H7. The first outbreak of *E. coli* O157:H7 was in 1982, start from then it has become widely known as Enterohemorrhagic *Escherichia coli* (Adamu *et al.*, 2014). Through previous study, this microbe have small infectious dose (Armstrong *et* al., 1996). The illness that can be caused by *E. coli* infection can range from mild diarrhea to bloody diarrhea to hemorrhagic colitis (HC) and life- threatening Hemolytic Uremic Syndrome (HUS) (Adamu *et al.*, 2014). In Malaysia, research done by Najiah *et* al (2010), found the presence of *E. coli* in mud crabs from Setiu Wetland. *E. coli* was extracted from gills and hepatopancreas of crabs from Ennore marine ecosystem which is a polluted area (Mahalaxmi *et al.*, 2013). In other research, pathogenic *E. coli* were also detected from seafood and the contamination was a secondary contamination (Kumar *et al.*, 2004). According to the international guidelines, the amount of *E. coli* in edible crustaceans should be between 10^6 to 10^7 (ICMSF, 1986).

2.2.2 *Vibrio cholerae*

Vibrio cholerae are gram negative bacteria that cause a life threatening diarrheal disease. Cholera outbreak all over the world has been the cause of consuming uncooked or raw seafood. Aquatic source such as estuaries and brackish water is one of the main sources of *V. cholera*. Like other *Vibrio* species, their growth are influence by environmental factors and their numbers are highest in warm water (Iwamoto *et al.*, 2010). The virulence factor of

V. Cholerae is colera enterotoxin (CT) which encoded by two hazardous genes forming the *ctxAB* operon (Koch *et al.*, 1993). A cholera outbreak was reported in one primary school in Kelantan in 2002 with 46 cases and 1 death. The primary source then was identified to be from contaminated food (MOH, 2006). In previous research, the presence of *Vibrio* in mud crabs collected from crab farms in India was consist of 5% from total mesophilic flora at 37 °C (Lalitha *et al.*, 2012). In Malaysia, *Vibrio cholerae was detected in* research on mud crabs sampled from Setiu wetland (Najiah *et* al., 2010). The risk of getting infection from *Vibrio* can be avoided cooking the seafood completely and by once the seafood is cooked, prevent cross contamination. Vibrio cannot be killed by freezing as it is an ineffective method (Ward *et al.*, 1997). *V. cholera* should not be present in raw crustaceans in order for them to be safely consume (ICMSF, 1986).

2.2.3 *Salmonella*

Salmonella are gram negative bacteria and a member of Enterobacteriaceae family. Infants, old adults and patients with immunodeficiency are prone to be infected with salmonelloses and it can transmit through consuming various foods (Drahovska *et al.*, 2001). Salmonellae able to reproduce in numerous environmental condition outside the living host. Although it can survive in various environment outside the living host, they are still sensitive toward heat and most of the time are killed at 70 °C and above (Pui *et al.*, 2011a). The most frequently relate with the cause of gastroenteritis is Salmonella typhimurium, which account for an estimation of 15% of all food-borne infection in the United States of America (Pui, *et al.*, 2011b) "*Salmonella* pathogenicity islands" (SPI) are certain area of the chromosome where abundance of *Salmonella* virulence factors are aggregated in (Kaur *et al*, 2012). In previous research, 15 out of 42 crabs collected from fishing harbor and fish retailers of Cochin, India was detected with presence of *Salmonella* (Kumar *et al.*, 2008). Fish and

shellfish can pick up *Salmonella* from polluted waters. In Malaysia, Hamdan *et al* (2008) found the presence of *Salmonella spp.* in short necked clam from East Coast, Malaysia. According to the suggested microbiological limits for raw crustaceans, *Salmonella* should not be present in them in order to be considered safe for consumption (ICMSF, 1986).

2.3 Crabs

Crabs are crustaceans, they are bottom-feeding omnivores. They inhabits the muddy bottoms, mangrove marshes, and river mouths in estuarine environments. They feed on primarily algae and also any other food such as mollusks, worms, other crustaceans, fungi and even bacteria. Mangrove crab to be precise, *Scylla serrata*, are large, tasty and relatively easy to catch, thus making them one of favorite seafood (Ewel, 2008). Scylla serrate can be found in the soft muddy bottom of estuaries and sheltered coastal habitat (FAO, 2010). Fig. 2.1 shows the morphology of *Scylla seratta*.

Although seafood are important source of protein, iodine, vitamin D, selenium and omega-3 oils, some safety precaution need to be taken so that foodborne illness can be avoid. Even in developing country such as Malaysia, the presence of choleragenic *Vibrio cholera* in domestically marketed seafood are reported (Elhadi *et al.*, 2004). Indicator organisms, usually coliforms, have been used as indicator of fecal contamination in crabs to ensure the safety of consuming this seafood.

The habitat of crabs has huge influence on the bacterial flora of them. Microorganisms that are present on their body surface or in the intestine may enter the crab meat and lead to contamination. Water, sediments and food may be the origin of the microbes that are present in their body parts (Aftabuddin *et al.*, 2013).

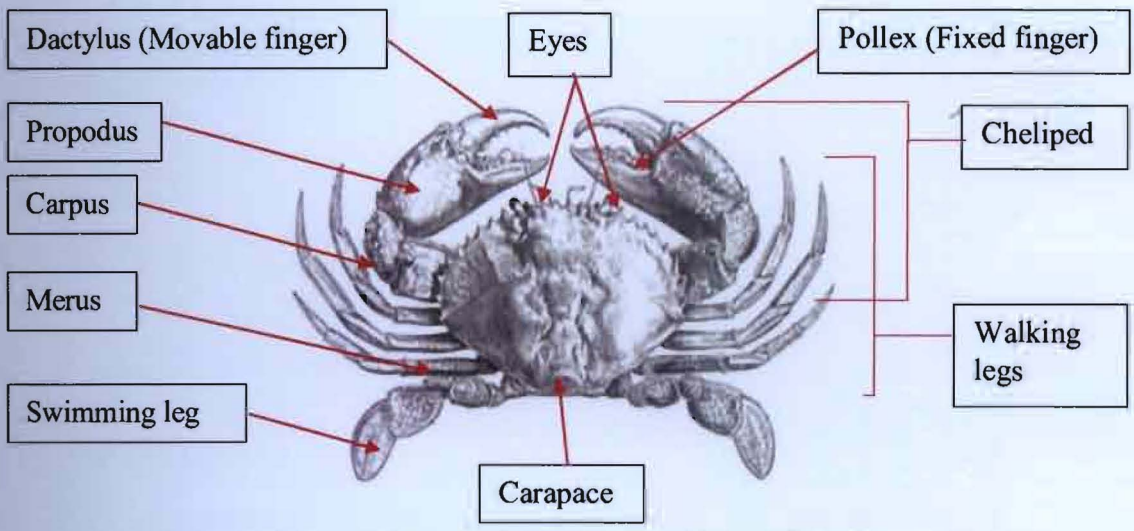

Figure 2.1 External anatomy of a mud crab (*Scylla serrata*) (FAO, 2010).

Previous research shows there are presence of bacteria in crabs that were collected near sewage and sludge disposal area (Grisi & Gorlach-Lira, 2010). In United States, crabs shrimp and oysters are the most seafood associated as sources of pathogens that naturally occur freshwater and marine environments. Most of the cases originate from eating under-cooked or raw shellfish (Cato, 1998). Research on crabs from Ennore seacoast Bay, Bengal north east coast of India shows that bacteria isolated from their tissues are mostly fecal coliforms which may cause by sewage contamination (Mahalaxmi *et al.*, 2013). In Malaysia, research on mud crabs sampled from Setiu wetland shows that the presence of *Vibrio cholera* and *Escherichia coli* inside them (Najiah *et al.*, 2010). Another research from Kerala, India, stated that farmed mud crab from there contain high level of enterococci and faecal coliforms (Lalitha *et al*, 2012). There have been reports on microbial quality of tropical freshwater fish and ponds in Sarawak, Malaysia but this information on crabs is limited (Apun *et al.*, 1999). Even in India, a country famous with their crab aquaculture industry, the presence of zoonotic disease is still not known and much research need to be done to understand the pathogens, host and environmental interaction of mud crabs (Jithendran *et al*, 2010).

3.0 MATERIALS AND METHOD

3.1 Materials

The list of materials is displayed in Appendix A.

3.2 Methodology

3.2.1 Sample Collection

Three sampling trip were done to collect mud crab, *Scylla serrata,* from the month of February to April 2015. The first sampling site is crab pond at Sematan, the second site is at Pendam River, Asajaya and the third source of sample is at Kota Samarahan wet market. The number of crab successfully obtained from the crab pond were three and from Pendam River were five crabs. The crabs were obtained using traps with chicken head as bait. Five mud crab sample from Kota Samarahan wet market was bought from the local vendor.

During each sampling trip except at Kota Samarahan Wet Market, the temperature and pH of the water were recorded using pH and temperature meter (pH Scan 30, Bante, Rome). Five dissolved oxygen bottles were used to collect water samples from each sampling sites in order to test for their Dissolved Oxygen and Biochemical Oxygen Demand using Dissolved oxygen meter (Pro 2030, YSI inc., USA). Water sample collection is as shown in Fig. 3.1. The result of Biochemical Oxygen Demand of water samples were obtained using the 5-day BOD test (Patil *et al.*, 2012). The parameters then were compared with the National Water Quality Standards for Malaysia (Zainudin. 2010).

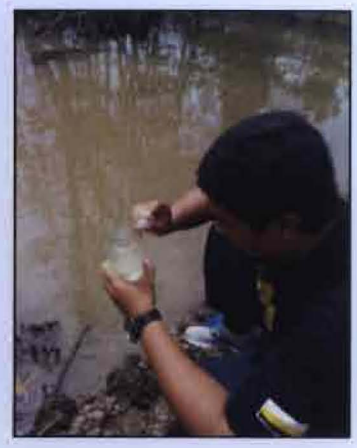

Figure 3.1 Water sample collection process at crab pond, Sematan.

3.2.2 Sample Processing

The mud crabs were all transferred from sampling site to the lab in an ice box with some water in it to maintain their freshness and to prevent death of the crabs. Upon arrival to the lab, the size and weight of the crabs were taken and recorded.

To kill a crab, first the crab was inverted to its back with its legs facing upward. When it facing upward, there are small pointed flap (Mahalaxmi *et al.*, 2013). The flap then was lift to expose the small hole in the shell. This hole was pierced down using a sharp object such as skewer until it hit the other side of the shell to cut their ventral nerve cord. The flesh were taken from their cheliped and walking leg part. To obtain the flesh from these parts, the shell was broken using a stone pestle. 30 gram of flesh were obtain from each crabs and then they were minced using sterilized knife. Each time of handling different crabs the knife and stone pestle were wiped with 70% ethanol and then flamed to prevent cross contamination. Fig. 3.2 shows the sample processing process.

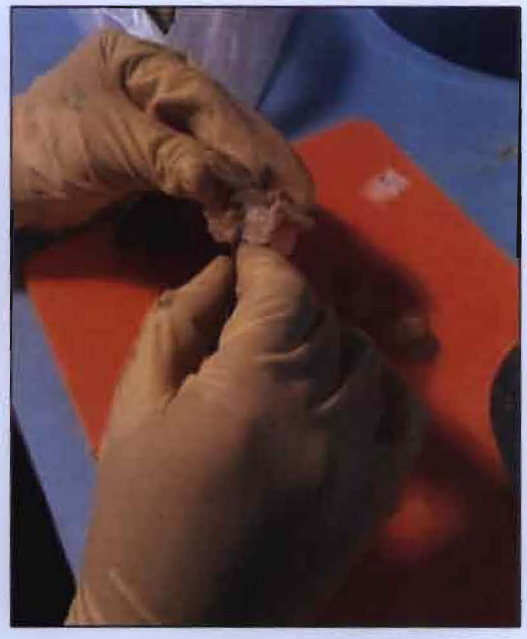

Figure 3.2 Crab flesh extraction process.

3.2.3 Enrichment Process

Thirty gram of flesh extracted from each crab were separated into three part of 10 gram each. Each part were enriched with 90 mL enrichment broth which are Selenite Cystine broth (OXOID, England), Luria-Bertani broth (OXOID, England), and Alkaline Peptone water (OXOID, England) in 250 mL conical flask. All the enrichment broth were prepared and sterilized by autoclaving at 121 °C for 15 minutes before going for sampling. The mixture were then shaken for a while to homogenize them. Later the mixture were then incubated inside an incubator at 37 °C for 24 hours.

3.2.4 Serial dilution

After incubation for 24 hours, the enriched samples were then undergo serial dilution using Alkaline Peptone water (OXOID, England) as diluent. Serial dilution of enriched samples were done until dilution 10^{-5}. Fig. 3.3 show the example of how serial dilution process was conducted in this project.

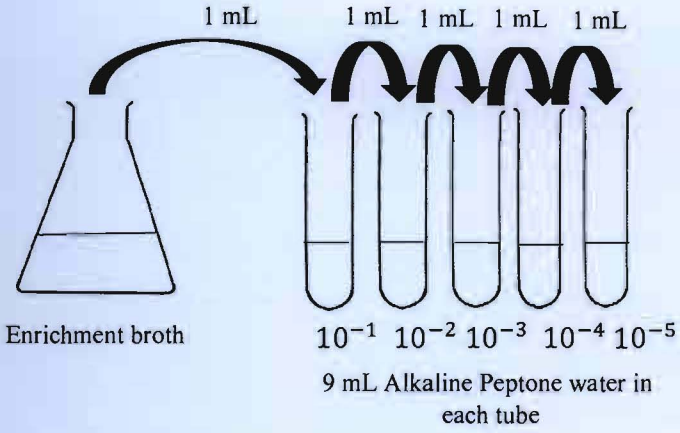

Figure 3.3 Serial dilution process.

3.2.5 Spread Plating and Bacterial Count

Right after serial dilution, dilution 10^{-3} until 10^{-5} were spread plated onto 3 different selective agar which were Thiosulphate-Citrate-Bile-salts-Sucrose agar (TCBS) (OXOID, England) for *Vibrio cholereae*, Xylose Lysine Deoxycholate agar (XLD) (OXOID, England) for *Salmonella spp.*, and Eosine Methylene Blue agar (EMB) (OXOID, England) for *Escherichia coli*. Later, they were incubated for 24 hours in a 37 °C incubator. After a period of 24 hours incubation, the number of colony formed on the selective agar were counted and reported as colony forming unit per mL (cfu/mL) (Sarnoski *et al.*, 2010). The equation below shows how cfu/mL were calculated:

$$\frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume plated (mL)}} = \text{CFU/mL}$$

After the colonies were counted, a colony was picked to be grown in Luria-Bertani broth and nutrient agar for biochemical test purposes, and storing in glycerol stock for long term storage. Gram staining were also conducted for morphological test (Hucker *et al.*, 1923). Their morphology were observed using a light microscope (BX51, Olympus, Japan).